SARTORIUS

Best Practice Guide

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Best Practice Guide: Octet® SF3 Assay Orientation

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Introduction

In surface plasmon resonance (SPR), irrespective of the chemistry used (coupled or captured) one binding partner is injected in solution (as analyte) over a specific binding partner, which is immobilized or captured on the sensor chip (as ligand).

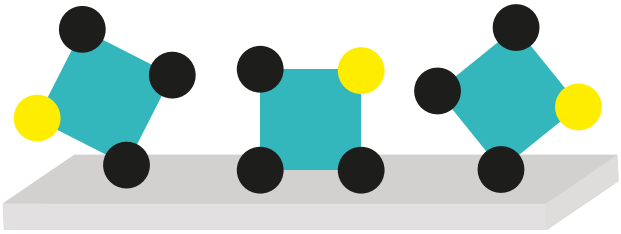
In general, assay orientation plays an important role in SPR assays and should be designed with the simplest biologically relevant model in mind. Oftentimes the inability to fit data to a simple model can be a result of assay design and not a systemic flaw in the technology.

The Impact of Different Immobilization and Capture Approaches

Although there are many types of chemistry available for covalently attaching proteins to SPR sensor chips, the most commonly used is amine coupling. Benefits of amine coupling include its stability and that the protein does not have to be modified in any way. In addition, the amount of ligand required is low and it is generally easy to achieve the same immobilization level in subsequent assays.

Many proteins have accessible primary amines on their surface, which means that numerous orientations of the protein can be covalently attached to the sensor chip surface. Therefore, the main drawback associated with amine coupling is the creation of a heterogenous sensor chip surface. As shown in the Figure 1, this can cause obstruction or blocking of binding sites. Additionally, low pH solutions that are required to drive the protein towards the chip surface during amine coupling can cause protein degradation. Another drawback associated with amine coupling is the need for high purity ligands as impurities, if amine bearing, can co-immobilise to the sensor chip surface and reduce the binding capacity.

Figure 1
Amine Coupling



Note. Primary amines (black dots) on the ligand can be covalently attached to carboxymethyl groups present on the sensor chip surface in multiple orientations, which can hinder or block the binding site (yellow dot).

It is important to consider that chemically biotinylated proteins can be captured in a similar 'random' orientation if they contain multiple primary amines. However even if the protein contains only one primary amine suitable for labeling then there is the possibility that capture via the biotin may occlude the binding site.

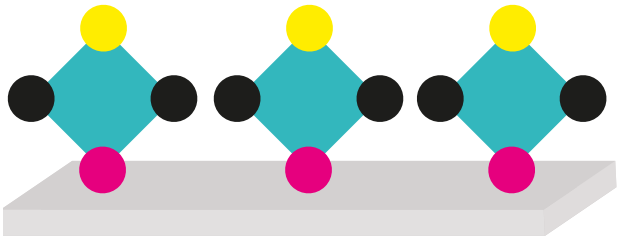
The most common solution to problems with amine coupling is to introduce an affinity tag into the ligand which allows a specific ligand orientation to be created, where all molecules are orientated in the same way, which helps prevent any differences in binding site presentation. In addition, unlike amine coupling which requires high purity reagents, affinity tags allow lower purity preparations to be used. During affinity capture, only those molecules bearing the affinity tag will be captured while impurities remain in the bulk flow and are removed under standard running buffer flow.

His-Tag

His-Tag, containing 6 or more Histidine residues at the N- or C-terminal, is one of the simplest and most frequently used in affinity purification and capture assays.

The most common way to capture a His-Tag ligand is using an anti-histidine antibody, which can detect mixed His-Tags (non-selective), which allows the use of one antibody for all assays, or antibodies that are selective against N- or C-terminal His-Tags. The antibody can easily be regenerated using a low pH Glycine solution and enables multiple ligand and analyte combinations to be tested.

Figure 2



Note. Inclusion of a specific affinity tag (magenta dot) such as His-Tag, AviTag™ or Fc Tag allows oriented capture of the ligand.

Another common way of capturing His-tagged molecules is by using a specialized sensor chip such as the Octet® SPR HisCap sensor chip (Catalog No. 19-0058). Based on the principle of Immobilized Metal Affinity Chromatography (IMAC), the His-tagged molecule is captured via Ni^{2+}/NTA chelation and regenerated using EDTA to strip the metal ions and the captured his-tagged protein away. The regenerated HisCap chip surface can then be re-used by recharging the NTA surface with fresh Ni^{2+}.

The main benefit of His-Tag capture is that the surface can be easily regenerated and, in general, can handle multiple regenerations before any decrease in capture efficiency or baseline drift is noted.

Biotin-Streptavidin
Biotin is a small molecule that has a very high affinity for streptavidin (Kd ~10^{-14} M), which can generate stable baselines for SPR assays. Labeling of molecules with biotin rarely interferes with function, making it a good choice for capture, although as discussed in the previous section, the surface presentation can be heterogenous.

Therefore, site specific biotinylation of ligands is highly desirable to not only create a homogenous sensor chip surface but also a highly stable ligand surface and baseline.

Many proteins are commercially available in large quantities and contain a site specific biotin molecule due to the increase in use of AviTag™ technology, which allows SPR users to create a homogenous sensor chip surface with an exceptionally stable baseline on streptavidin prepared sensor chips such as the Octet® SPR SADH sensor chip (Catalog No. 19-0130).

Unlike His-Tag capture, regeneration can become an issue and careful consideration must be given to choose a suitable regeneration solution.

Fc Tag
IgG-Fc tags have been shown to improve the stability of proteins and increase the solubility of the yield and are therefore, a popular choice of affinity tag for commercial proteins.

The use of Fc Tag as a default tag presents challenges for SPR assays as, although the Fc tag allows assay-specific orientation through capturing via the Fc tag, measurement of Fc containing proteins, such as IgG antibodies, becomes challenging, as they also contain the structural motif that binds to the Fc-tagged capture molecule. Inversion of the assay, where the antibody is captured using a capture molecule such as protein A or G also causes the same issue, as the analyte with an Fc tag will bind to the capture molecule causing a large amount of undesirable binding. Standard techniques to reduce non-specific binding cannot be used as there is a specific capture molecule there for the Fc region, which will also affect ligand capture.

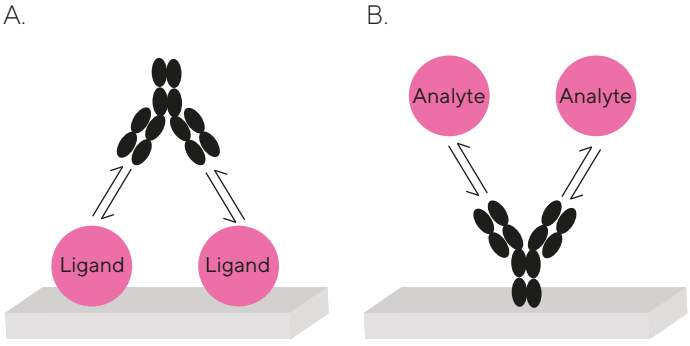
Therefore, careful consideration must be given to the assay setup when proteins containing Fc Tags are required for creating a homogenous sensor chip surface.

Orient Assays to Avoid Avidity

Creation of a homogenous ligand bound sensor chip surface is desirable but other issues such as avidity must be considered when designing SPR assays. The conventional bivalent IgG homodimer antibody presents two independent and equal binding sites for a specific antigen, and therefore, is susceptible to avidity effects if assay orientation is not considered.

As shown in Figure 3A, when the antibody is injected as the analyte, each binding site has the potential to bind to the ligand and cause avidity issues.

Figure 3



Note. Assay orientation choice plays an important part in determining accurate kinetics and affinity.

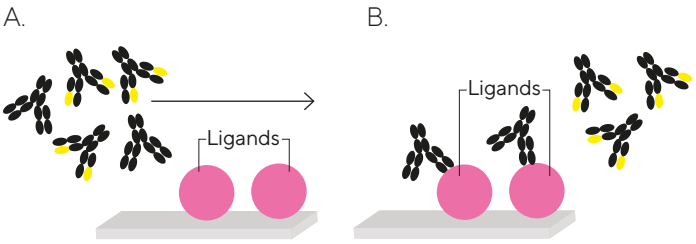
Therefore, it is usually advisable to inject the antigen over the immobilized antibody (Figure 3B) to avoid avidity artifacts, provided that the antigen is monovalent.

This assay format offers the advantages of both keeping the antigen in solution to enable access to all epitopes, and scalability, with the Octet® SF3 allowing a large number of ligands and antigens to be analyzed per assay array, allowing hundreds of interactions to be analyzed in parallel.

Structure Function Relationships
Samples stored under stress conditions such as, heat, acid/base hydrolysis and oxidation are often used in structure function relationship studies. Assay orientation can become especially important when looking at stressed samples, as assay orientation may cause the assay to be insensitive to stress or function alterations due to preferential binding of the non-stressed molecules over the weaker binding stressed molecules.

For example, in Figure 4A there is a heterogenous population of stressed molecules (stress is shown in yellow) as may be found in a short-term stress study. If the antibody is the analyte and the antigen is the ligand then the un-stressed antibodies will preferentially bind over the stressed molecules (Figure 4B); therefore, the assay is insensitive to measuring the effect on stressed molecules and may lead to an inaccurate conclusion from the data.

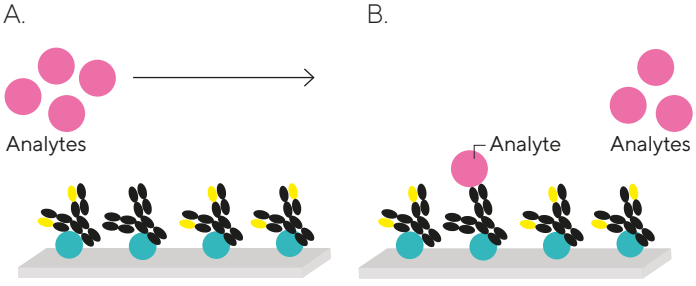
Figure 4



Note. Unstressed molecules (all black) preferentially bind to the target when compared to stressed molecules (yellow), which may lead to incorrect conclusions being reached.

In a modified assay setup with the target as the analyte (Figure 5A), the assay sensitivity to stress or function alterations is improved due to the non-biased presentation of the molecule of interest. In this orientation the sensor chip surface heterogeneity represents the stressed molecule population more accurately and a decreased response would be observed compared to only unstressed molecule being captured. This assay orientation also offers the advantage of decreasing avidity effects as described above.

Figure 5

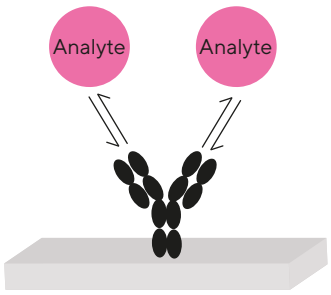


Note. Capture-based assays may offer a truer representation of stress effects.

Calculating Surface Activity

It is desirable to determine the activity of the captured or immobilized ligand (and as such, its conformational structure) during assay development. There is a direct 1:1 relationship between the amount of ligand and the observed Rmax in SPR assays. For a captured antibody (Figure 6) this is due to an increased number of antigen binding sites as more mAb is immobilized and as such can bind more analyte, which leads to an increase in the observed Rmax. (Rmax vs Immobilization Level).

Figure 6



Note. Captured antibodies present two independent and equal binding sites for a specific antigen.

To calculate the surface activity a range of immobilization levels must be assessed, and the mass of the ligand and analyte known. As an example, in Figure 6, the mass of the mAb is 75 kDa per antigen binding site and the mass of monomeric analyte is 30 kDa.

In an assay setup where 100% of the surface binding was available to the analyte a molar ratio of 0.40 (30 kDa/75 kDa) would be observed. Comparing the gradient of the Rmax vs Immobilization Level to the predicted molar ratio allows the user to determine the surface activity. For example, if an Rmax vs Immobilization Level gradient of 0.2 was determined then a surface activity of 50% is observed (0.2/0.4*100).

As discussed above, it must be remembered that standard immobilization techniques such as amine coupling create a heterogenous surface and therefore, will affect surface activity.

Conclusion

Consideration of orientation during assay design can help create homogenous surfaces that minimize avidity artifacts and allow data to be biologically relevant and fitted to the simplest model possible.

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